Molecular Mechanisms Optimizing Photosynthesis During High Light Stress in Plants

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To my family
Molecular Mechanisms Optimizing Photosynthesis
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ABSTRACT: Oxygenic photosynthesis is the process by which plants, algae and cyanobacteria use solar energy to convert water and carbon dioxide into molecular oxygen and carbohydrates. Photosynthesis sustains life on Earth since it provides not only energy for individual growth, but also represents the starting point of the food chain for most living organisms. Sunlight is essential for driving photosynthesis, but it is also known that in excess it can be stressful with severe consequences for plant growth. In this thesis I have used the model plant Arabidopsis thaliana to study molecular mechanisms optimizing photosynthesis during high light stress.

One of these mechanisms is the reversible phosphorylation of proteins in the water-oxidizing photosystem II (PSII) complex. The serine/threonine-protein kinases STN7 and STN8 are involved in the phosphorylation of the PSII light-harvesting complex (LHCII) and core proteins, respectively. In Paper II, I found variation in the phosphorylation levels of these proteins in Arabidopsis natural accessions. In high light conditions, I found a correlation between the STN8 protein abundance and the D1 protein phosphorylation level. In growth light conditions, D1 and LHCII phosphorylation correlated with longitude and in the case of LHCII phosphorylation with temperature variability as well.

Another molecular mechanism for plants to overcome high light stress is via PSII repair. STN8-mediated PSII core phosphorylation is an early and crucial step for efficient PSII repair, since it alters the folding of the thylakoid membrane in a manner facilitating lateral migration of complexes to the sites of repair. Among three laboratory Arabidopsis accessions studied, Ws-4 displayed a reduced STN8 level resulting in decreased PSII core protein phosphorylation (Paper I). Nevertheless, the downstream steps in PSII repair proceeded normal or slightly faster. This phenomenon is probably due to compensatory mechanisms involving additional lipids and carotenoids to increase membrane fluidity and thus lateral migration of complexes.

The thylakoid ATP/ADP carrier (TAAC) transports ATP into the thylakoid lumen for nucleotide-dependent reactions. In Paper III, I have found that TAAC-deficient plants displayed wild-type levels of PSII protein phosphorylation but slower disassembly of complexes and slower D1 protein degradation. I propose that ATP supplied by TAAC into the lumen is used for nucleotide-dependent reactions with roles in various steps of PSII repair. I have also found that, via its transport activity, TAAC may consume part of the proton gradient across the thylakoid membrane, which is critical for the initiation of photoprotective mechanisms.

In a proteomics study of the stroma thylakoid membrane from Arabidopsis (Paper IV), I identified 58 proteins, including previously known ones as well as new putative thylakoid proteins with roles in photosynthesis transport, translation, protein fate, metabolism, stress response and signaling. This thesis deepens our understanding of photosynthetic regulation at the molecular level and improves the biochemical overview of the chloroplast thylakoid membrane.

Keywords: high light stress, natural variation, photosynthesis, protein phosphorylation, photoprotection, photosystem II, proteomics, STN kinase, thylakoid membrane, TAAC
LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numerals in the text:

   Photosystem II function and dynamics in three widely used *Arabidopsis thaliana* accessions. PLoS One 7(9): e46206

II. **Flood PJ¹, Yin L¹, Herdean A, Harbinson J, Aarts MG, Spetea C (2014)**


IV. **Yin L, Vener AV, Spetea C.**
    Proteomic study of stroma thylakoid membranes from *Arabidopsis thaliana*. Manuscript.

¹ Shared first authorship
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Chl</td>
<td>Chlorophyll</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced fragmentation</td>
</tr>
<tr>
<td>Col-0</td>
<td>Columbia-0</td>
</tr>
<tr>
<td>CP43</td>
<td>Photosystem II chlorophyll apoprotein 43</td>
</tr>
<tr>
<td>D1</td>
<td>Photosystem II D1 protein</td>
</tr>
<tr>
<td>ESI</td>
<td>Electronspray ionization</td>
</tr>
<tr>
<td>ETD</td>
<td>Electron transfer dissociation</td>
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<tr>
<td>FD</td>
<td>Ferredoxin</td>
</tr>
<tr>
<td>FQR</td>
<td>Ferredoxin-plastoquinone reductase</td>
</tr>
<tr>
<td>GL</td>
<td>Growth light</td>
</tr>
<tr>
<td>HL</td>
<td>High light</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>Ler-0</td>
<td>Landsberg erecta-0</td>
</tr>
<tr>
<td>LHCII</td>
<td>Light-harvesting complex of photosystem II</td>
</tr>
<tr>
<td>MGDG</td>
<td>Monogalactosyl diacylglycerol</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NPQ</td>
<td>Non-photochemical quenching</td>
</tr>
<tr>
<td>PS</td>
<td>Photosystem</td>
</tr>
<tr>
<td>PBCP</td>
<td>photosystem II core phosphatase</td>
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<tr>
<td>PsbO</td>
<td>Oxygen-evolving enhancer protein 1</td>
</tr>
<tr>
<td>PSI</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>Photosystem II</td>
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<tr>
<td>STN</td>
<td>Serine/threonine-protein kinase</td>
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<tr>
<td>TAAC</td>
<td>Thylakoid ATP/ADP carrier</td>
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<tr>
<td>TAK</td>
<td>Thylakoid-associated kinase</td>
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<tr>
<td>TAP38/PPH1</td>
<td>Thylakoid phosphatase 38</td>
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1. INTRODUCTION

1.1 Photosynthesis – a preface

Oxygenic photosynthetic organisms such as cyanobacteria, algae and plants use sunlight as the energy source to convert \( \text{H}_2\text{O} \) and atmospheric \( \text{CO}_2 \) into carbohydrates and molecular oxygen according to the following simplified equation:

\[
\text{H}_2\text{O} + \text{CO}_2 + \text{sunlight} \rightarrow (\text{CH}_2\text{O}) + \text{O}_2
\]

These carbohydrates provide energy directly or indirectly via food chains to living organisms on Earth, including humans. The oxygen produced is released into the atmosphere and is essential for all aerobic life forms.

Pre-historical algae sedimented and over time converted into the fossil fuels we use today. The use of fossil fuels has a limit, since the production spanned millions of years, thus alternative energy sources are needed. Artificial photosynthesis has become an attractive option. It mimics natural photosynthesis, but it uses solar energy to produce biofuels in the form of lipid-rich membranes or molecular hydrogen. The simplified equation for artificial photosynthesis can be presented as follows:

\[
\text{H}_2\text{O} + (\text{CO}_2) + \text{sunlight} \rightarrow \text{(biofuel)} / \text{H}_2 + \text{O}_2
\]

Thus, a deeper understanding of the basic and regulatory aspects of photosynthesis becomes an important agenda for both food and energy reasons. My thesis work contributes to the further understanding of the molecular mechanisms behind the optimization of photosynthesis during abiotic stress conditions such as high-intensity light (known as high light, HL).

1.2 Chloroplast structure, composition and function

In eukaryotic photosynthetic organisms, photosynthesis takes place in the green organelle named the chloroplast (Figure 1). It has an outer and an inner envelope membrane enclosing the soluble stroma. A third membrane system called the thylakoid membrane is situated in the stroma. Further inside the thylakoid membrane is a soluble space called the thylakoid lumen. The different compartments of the chloroplast have several distinct functions. The envelope membranes are involved in the exchange of solutes and organic compounds between the stroma and the rest of the cell, and are
also the site of import for nuclear-encoded chloroplast proteins. The stroma is where carbon fixation occurs, but also biosynthesis of various compounds and the synthesis of chloroplast-encoded proteins. The thylakoid membrane harbors pigment-protein complexes involved in light harvesting, water oxidation, electron and H⁺ transfer to form chemical energy (ATP, NADPH). The thylakoid lumen is involved in the regulation of the above reactions and in signaling events.

![Figure 1. Schematic overview of chloroplast structure. Outer and inner envelope membranes enclose the soluble stroma. The thylakoid membrane is situated in the stroma and encloses the soluble lumen. The thylakoid membrane consists of stacked grana region and unstacked stroma lamellae regions.](image)

Thylakoid membranes contain two types of membrane regions: cylindrical stacks, comprised of multiple tightly appressed layers, called grana, and unstacked interconnecting single-membrane regions, called stroma lamellae (Anderson and Andersson 1988; Nevo et al. 2012). The stacked and unstacked regions comprise 80% and 20%, respectively, of the membrane surface (Albertsson 2001). Photosynthetic complexes are non-uniformly distributed over the thylakoid membrane. Photosystem II (PSII) and its light-harvesting complex II (LHCII) are mainly located in grana stacks, whereas most of the PSI, LHCI and H⁺-translocating ATP synthases are in the unstacked membrane regions. Cytochrome b₆f complexes are distributed evenly between the two types of thylakoid membranes (Andersson and Anderson 1980; Albertsson et al. 1990). This phenomenon is known as lateral heterogeneity of the thylakoid membrane.

The dynamics in the lateral distribution of photosynthetic complexes play an important role in their biogenesis, function, regulation and repair (Kirchhoff 2014). Especially under high light conditions, fluidity of the thylakoid membrane was suggested to be crucial for the photodamaged protein complexes to be able to migrate from grana to stroma-exposed lamellae for protein degradation and subsequent replacement (Aro et al. 2005; Yamamoto et al. 2014).
1.2.1 Photosynthetic complexes

1.2.1.1 Photosystem II

Photosystem II (PSII) is considered to be ‘the engine of life on Earth’, due to its ability to utilize water molecules as a source of electrons. Active PSII functions in a dimeric form, where each monomer contains more than 20 protein subunits. In addition to proteins, 35 chlorophylls, two pheophytins, 11 β-carotenes, over 20 lipids, and two plastoquinones were found in the latest PSII crystal structure (Umena et al. 2011). Among the identified lipids were six monogalactosyldiacylglycerol (MGDG), five digalactosyldiacylglycerol (DGDG), four sulfoquinovosyldiacylglycerol (SQDG) and five phosphatidylglycerol molecules.

The main electron cofactors are the following: Mn$_4$CaO$_5$, a cluster which splits water into protons (H$^+$), electrons (e$^-$), and O$_2$; Tyr, a tyrosine molecule, which shuttles electrons to the "reaction center" of PSII; Chl P680, the reaction center pair of chlorophyll $a$ molecules, which is the primary electron donor of PSII; Pheo, a pheophytin molecule, which is the primary electron acceptor of PSII; plastoquinone Q$_A$, which is the primary stable electron acceptor of PSII; plastoquinone Q$_B$, which becomes mobile after accepting two electrons and called PQ (Orr and Govindjee 2010).

The PSII core complex consists of a large number of intrinsic and extrinsic subunits (Pagliano et al. 2013; Vinyard et al. 2013). The reaction center (RC) of PSII, defined as the minimum set required to oxidize water, is composed of the core integral subunits D1 and D2 binding most electron cofactors, and the inner antenna proteins CP43 and CP47 as well as the $\alpha$ and $\beta$ subunits (PsbE and PsbF) of cytochrome $b_{559}$ (Cyt$b_{559}$). They are preserved in all oxygenic photosynthetic organisms, and plastid-encoded in algae and plants. Besides this, the oxygen-evolving complex (OEC) containing Mn$_4$CaO$_5$ cluster is extrinsically attached with the lumenal side of PSII and associated with four subunits in plants: PsbO (the 33 KDa manganese stabilizing protein), PsbP (23 KDa) and PsbQ (11KDa), PsbR (10 KDa). In Arabidopsis, PSII complexes harboring the PsbO1 isoform have a higher oxygen-evolving activity than those with the PsbO2 isoform (Murakami et al. 2005); PsbO2 being hypothesised to regulate dephosphorylation and turnover of the D1 protein (Lundin et al. 2007a). PsbO is thought to be a GTPase with a role in the regulation of PSII repair (Lundin et al. 2007b). PsbP plays an important role for the assembly and/or stabilization of PSII-LHCII supercomplexes (Yi et al. 2007; Ido et al. 2009). PsbR has been suggested to stabilize PSII Mn cluster also(De Las Rivas et al. 2007; Allahverdiyeva et al. 2013).

In the PSII dimeric form, the core complex interacts with LHCII proteins (Lhcb1–6). Lhcb1–3 form trimers in various compositions. Strongly bound $S$ trimers interact with the PSII core via monomeric antenna proteins Lhcb5 (also called CP26), which form C252; in addition to that, two moderately bound LHCII trimers (M2) interact with the core via Lhcb4 (CP29) and Lhcb6 (CP24) to form PSII-LHCII supercomplexes (Kouril et al. 2012).
1.2.1.2 *photosystem I*

The PSI complex mediates light-driven electron transfer from cytochrome $b_{6f}$ via plastocyanin to the ferredoxin-NADP$^+$-complex. In plants, the PSI complex consists of at least 19 protein subunits, approximately 175 chlorophyll molecules, two phytolquinones and three Fe$_3$S$_4$ clusters (Ben-Shem et al. 2003). The predominant type of PS I in peas (*Pisum sativum* var. *Alaska*) consists of 15 core subunits and four peripheral light-harvesting (LHCI) proteins organized as two heterodimers, Lhca1/Lhca4 and Lhca2/Lhca3. Only PsaA, -B, and -C, out of the 15 core proteins, are directly involved in binding the electron transport cofactors P700 (a chlorophyll dimer), $A_0$ (a chlorophyll $a$ molecule), $A_1$ (a phytolquinone), $F_4$ (a [4Fe–4S] iron–sulfur cluster), $F_8$ and $F_9$ (both [4Fe–4S] iron–sulfur clusters). The other core proteins perform additional functions: PsaD and PsaE provide the docking site for ferredoxin on the stromal side of the thylakoid membrane; PsaF and PsaN are essential for the interaction with the luminal electron donor plastocyanin, and PsaF is important for the binding of the Lhca1/Lhca4-dimer (Jensen et al. 2007).

The core antenna is composed of about 100 Chl $a$ and 20 $\beta$-carotene molecules (Jordan et al. 2001; Ben-Shem et al. 2003). Most of the Chl and carotene molecules are bound to the main subunits PsaA and PsaB. In the case of LHCl, Lhca1-4 binds 13 Chl, but Lhca1/3 binds three carotenoid molecules per polypeptide, whereas Lhca2/4 binds two carotenoids, mainly lutein and violaxanthin.

1.2.1.3 *Cytochrome $b_{6f}$*

The cytochrome $b_{6f}$ complex acts as the redox link between the photosynthetic reaction centers II and I and also functions in proton translocation (Cramer et al. 1996; Tikhonov 2013a). This complex functions as a dimer, with each monomer consisting of eight or nine subunits in cyanobacteria and plants, respectively (Baniulis et al. 2011). These consist of four large subunits: cytochrome $f$ with a c-type cytochrome, cytochrome $b_6$ with a low- and high-potential heme group, Rieske iron-sulfur protein containing a [2Fe-2S] cluster, and subunit IV. In addition to these, four small subunits (3-4 kDa) are present: PetG, PetL, PetM, and PetN, whose functions are unclear but might have critical roles in the assembly and/or stability of the complex. The plant-specific subunit is ferredoxin: NADP$^+$ reductase (FNR), with a physiological role to catalyze the final step of photosynthetic electron transport, namely the synthesis of NADPH for downstream carbon fixation (Mulo 2011).

1.2.1.4 *ATP synthase*

The chloroplast ATP synthase has two distinct components, CF$_1$ and CF$_0$. CF$_1$ is an extrinsic membrane protein subcomplex that contains the catalytic site(s) for reversible ATP synthesis. CF$_0$ is an integral membrane protein complex, which is responsible for
the conversion of an electrochemical proton gradient into rotational motion (Richter et al. 2005; Poetsch et al. 2007). CF₁ has a subunit stoichiometry of α₃β₃γδε (Süss and Schmidt 1982). CF₀ contains four different subunits named I, II, III₁₄ and IV (Poetsch et al. 2007). The III₁₄ oligomer forms the proton-driven rotor, and the rotating central stalk in the ATP synthase consists of subunits γ and ε. A second stalk, is composed of subunits I, II and δ, connecting cF₁ and cF₀ at the periphery and stabilizing the rotating machinery as a stator (Poetsch et al. 2007).

1.2.2 Chloroplast stroma and thylakoid lumen

1.2.2.1 Chloroplast stroma

The chloroplast stroma is the space between the inner envelope membrane and the thylakoid membrane. It contains chloroplast nucleoids, ribosomes, starch and many proteins involved in biosynthetic reactions. The most abundant protein in the chloroplast stroma is Rubisco, an enzyme that assimilates CO₂ into carbohydrates, during the called Calvin-Benson cycle. The stromal pH can reach 8.0 as compared to cytoplasmic pH in the range 7.0-7.5 during illumination (Wu and Berkowitz 1992; Hauser et al. 1995).

1.2.2.2 Thylakoid lumen

The thylakoid lumen contains not only OEC proteins, the electron carrier plastocyanin (PC) and photoprotection related violaxanthin de-epoxidase, but also up to 80 proteins in Arabidopsis (Peltier et al. 2002; Schubert et al. 2002; Kieselbach and Schroder 2003). Most of the luminal proteins have a role in the regulation of thylakoid biogenesis, activity and turnover of PSII and of NAD(P)H dehydrogenase (NDH)-like complexes (Jarvi et al. 2013). The role of a large proportion of luminal proteins is unknown. The luminal proteins, including PsbP, PsbR abd PsbQ, can be regulated by reversible phosphorylation using ATP imported by the thylakoid ADP/ATP carrier (TAAC) (Spetea and Lundin 2012). The redox thiol/disulfide modulation is also considered as an important regulation of luminal protein function (Jarvi et al. 2013). Lumen size becomes large in high light conditions with pH dropping from 7.0 in darkness to 5.8-6.5 in the light (Kramer et al. 1999; Cruz et al. 2001; Tikhonov 2013b).

1.3 Photosynthetic electron transport

The photosynthetic apparatus has developed a series of adaptive mechanisms to various light intensities, as linear and cyclic electron transfer through the thylakoid membrane occurs (Figure 2).
**Figure 2.** Organization of photosynthetic complexes, simplified linear and cyclic electron transport chains in the thylakoid membrane. Dashed red, blue and solid black lines represent the linear, cyclic electron and proton transport, respectively.

### 1.3.1 Linear electron transport

Linear electron transfer involves three major complexes, namely PSII, cyt$b$$_{6}$f and PSI. Electrons are first extracted from P680 with the help of solar energy, and transferred to the primary electron acceptor pheophytin. The Mn$_4$CaO$_5$ cluster extracts electrons from water and re-oxidizes P680. Pheophytin delivers electrons to plastoquinone Q$_{A}$ and then to Q$_{B}$, the later one being recycled in the PQ pool. PQ receives two H$^{+}$ from the stroma, becomes a mobile carrier that transfers electrons to Cyt$b$$_{6}$f and then releases H$^{+}$ into the lumen. Some of the electrons from Cyt$b$$_{6}$f are carried further on to plastocyanin (PC) through PSI and ending in NADP$^{+}$. The other electrons from Cyt$b$$_{6}$f are recycled back to the PQ pool through several electron carriers called the ‘Q cycle’. The H$^{+}$ concentration difference between the stroma and the lumen forms a proton motive force (pmf), which later on is used to produce ATP on the stromal side of thylakoids via the activity of ATP synthase.

As in the case of PSII, electrons are extracted from PSI P700 with the help of light energy. PC is a mobile electron carrier, through which electron are passed from cyt$b$$_{6}$f to PSI to reoxidize P700. The electrons are further carried to ferredoxin (Fd) in the stroma, ultimately reaching NADP$^{+}$, with these reactions catalyzed by ferredoxin: NADP$^{+}$ reductase (FNR).
1.3.2 Cyclic electron transport

The electrons reaching PSI can be sent back to the linear electron transfer by cyclic electron transfer. There are several pathways: a) plastoquinone reductase (NDH) regulated pathway, when electrons are transferred back to plastoquinone via NDH (Battchikova and Aro 2007; Peng and Shikanai 2011); b) electrons are delivered from ferredoxin (FD) to PQ via a putative protein ferredoxin-plastoquinone reductase (FQR) (Cleland and Bendall 1992); c) electrons are carried from FD to Cytb6f, and from there back to PQ. Cyclic electron flow does not produce O2 nor NADPH, but pumps H+ ions across the membrane, forming a proton motive force that can be used to power ATP synthase. ATP and NADPH are utilized in carbon fixation reactions in the stroma resulting in building blocks for carbohydrates, amino acids, lipids, etc.

1.4 Mechanisms optimizing photosynthesis during high light stress

1.4.1 PSII photoprotection

Plants have developed intricate internal defense mechanisms, also known as photoprotection, to cope with intense light conditions. In a rather broad definition, it can be considered that plants respond to prevent damage that can be caused by the accumulation of high levels of reactive oxygen species (ROS) by interactions between lipids, nucleic acids and proteins (Apel and Hirt 2004; Triantaphylides and Havaux 2009). Photoprotection can be executed to either reduce the absorption of light, by dissipating the excess light or by scavenging the ROS that are eventually produced. A photoprotective response involving the dissipation of excess light is known as non-photochemical quenching (NPQ). During this mechanism, the concentration of excited 3Chl* in LHCII is decreased by the activation of a heat dissipation channel, thus limiting 3Chl* formation (Müller et al. 2001; Cazzaniga et al. 2013).

NPQ has several components known as qE, qZ, qT, qI and qM. The fastest component, which is formed within 10-200 sec, is called qE and is fully dependent on thylakoid lumen acidification (Horton et al. 1996; Nilkens et al. 2010). Low lumenal pH activates the xanthophyll cycle, i.e., the conversion of violaxanthin to zeaxanthin (Yamamoto and Kamite 1972; Jahns et al. 2009). This NPQ component also involves the LHC-like subunit PsbS that senses low lumenal pH through two lumen residues that can accept protons (Li et al. 2000; Li et al. 2004). The acceptance of protons by PsbS and the binding of zeaxanthin to PSII causes conformational changes in the antennae that result in enhanced thermal dissipation of excitation energy. qZ was recently resolved strictly as a zeaxanthin-dependent NPQ component, which is formed within 10-30 min and is independent of PsbS (Nilkens et al. 2010). The photoinhibitory component (qI) takes longer (> 30min) to form, and is much less characterized than qE because of a mix of photoprotection and photodamage roles (Müller et al. 2001; Nilkens et al. 2010). The
qT component relaxes within minutes and is important for algae during state transition, but less so for photoprotection in plants (Niyogi 1999). Besides all these dissipative mechanisms/components located within the chloroplast, plants can move chloroplasts within the cell to avoid damage from over-excitation energy. The component involved in NPQ is called qM. A mutant deficient in chloroplast avoidance movement formed half of NPQ compared with wild type plants during illumination (Cazzaniga et al. 2013).

1.4.2 PSII photoinhibition: damage and repair

Abiotic and biotic stresses are the two major causes of reduction in plant growth in nature. For major crops, more than 50% reduction in the average yield is due to abiotic stresses (Navabpour et al. 2003). One abiotic stress, high-intensity/excessive light is the major cause of photosynthetic apparatus damage/inactivation (Barber and Andersson 1992; Tyystjärvi and Aro 1996; Kanervo et al. 2005). If irreversible, this process is known as photoinhibition and often leads to reduced plant growth and fitness. Plants have developed a rapid and efficient repair system to recover from the damage, named PS II repair (Aro et al. 1993; Tyystjärvi and Aro 1996; Melis 1999; Kanervo et al. 2005; Nixon et al. 2010). To measure the extent of inhibition by light (PSII inhibition), it is essential to determine the balance ratio between photodamage and repair (Murata et al. 2007).

One classical model is that PSII damage happens via a two-step process: the first step is the light-dependent destruction of the Mn$_4$CaO$_4$ cluster, which is slow and rate-limiting; the second step is the inactivation of the photochemical reaction center of PSII by light that has been absorbed by chlorophyll, which is fast (Ohnishi et al. 2005). Another classical model is that inefficiency of electron transport at the PSII acceptor side will cause formation of P680 triplet, which can interact with oxygen and form ROS. These ROS then oxidize the D1 amino acids and cause damage (Vass et al. 1992; Napiwotzki et al. 1997). A third model is one-step photodamage of the reaction center of PSII by ROS (Murata et al. 2007). ROS have also been shown to inhibit PSII repair, especially the synthesis of the D1 protein, rather than only accelerating photodamage to PSII (Nishiyama et al. 2001). Thus, ROS increase the extent of photoinhibition by inhibiting the repair of PSII.

The PSII repair cycle is a multi-step process (Figure 3) (Aro et al. 1993; Melis 1999; Baena-Gonzalez and Aro 2002; Nath et al. 2013a): 1. phosphorylation of PSII core proteins by STN8 kinase and monomerization of the PSII complex; 2. relocation of damaged PSII monomer towards stroma lamellae and dephosphorylation of PSII core proteins by PBCP phosphatase; 3. dissociation of CP43 and oxygen evolving complex proteins and the degradation of photo-damaged D1 protein by specific proteases (see below); 4. de novo D1 synthesis and insertion into the thylakoid membrane; 5. activation, reassembly and migration to the grana to form a newly functional PSII dimeric complex.
It has been suggested that the rate of D1 degradation is the major rate-limiting step of the PSII repair process (Melis 1999). There are several proteases involved in the degradation of photodamaged D1 protein. Chloroplast ATP-dependent oligomeric metalloprotease FtsH has been shown to be involved in D1 protein degradation (Nixon et al. 2005; Komenda et al. 2006; Yoshioka and Yamamoto 2011). Besides FtsH, Deg1 is involved in D1 degradation, yielding C-terminal 16 kD and 5.2 kD products (Kapri-Pardes et al. 2007). Deg2 protease also can cleave the photodamaged D1 in the stroma-exposed DE-loop (Haußühl et al. 2001) yielding N-terminal 23kD and C-terminal 10kD fragments (Huesgen et al. 2009). Great efforts have recently been made to identify PSII repair cycle related kinases, phosphatases, proteases and a large array of luminal proteins, but the PSII repair regulatory pathway is still far from completely known. (Jarvi et al. 2013; Nath et al. 2013b).
1.4.3 Chloroplast reversible protein phosphorylation

As a major post-translational modification, reversible protein phosphorylation plays an important role in the regulation of photosynthesis. The structure of a protein can be conformationally changed by the addition or removal of a phosphate \( \text{PO}_4^{3-} \) group to a polar R group of an amino acid residue, since the hydrophobicity of a protein is altered by this modification. As a result, phosphorylation catalyzed by kinases activates many proteins, contrary to dephosphorylation catalyzed by phosphatase, which deactivates proteins. The first evidence for chloroplast protein phosphorylation was provided by Bennett in 1977, because radioactively labelled \(^{32}\text{P}\) was incorporated in certain amounts into chloroplast proteins (Bennett 1977). Recent large-scale mass spectrometric analyses in the chloroplast have expanded its phosphoproteome from a few proteins to around 200 (Sugiyama et al. 2008; Baginsky and Gruissem 2009; Lohrig et al. 2009; Reiland et al. 2009).

In thylakoid membranes protein phosphorylation is regulated by the redox state of photosynthetic complexes. Changes in thylakoid proteins can regulate photosynthesis in response to environmental conditions, including light, temperature and drought stress conditions (Vener et al. 1998; Aro and Ohad 2003). Recent research has been focused on two serine/threonine protein kinases, 7 and 8 (STN7 and STN8), and on the newly discovered thylakoid phosphatase 38 (TAP38/PPH1) and photosystem II core phosphatase (PBCP), because of their ability to phosphorylate/dephosphorylate thylakoid photosynthetic proteins in Arabidopsis (Bellafiore et al. 2005; Bonardi et al. 2005; Vainonen et al. 2005; Pribil et al. 2010; Shapiguzov et al. 2010; Pesaresi et al. 2011; Samol et al. 2012). The STN7 and STN8 kinases were traced from homologues Stt7 and Stt1 in green alga (Depege et al. 2003).

1.4.3.1 STN8-mediated phosphorylation

The STN8 kinase is known to phosphorylate the PSII core subunits D1, D2, CP43 and PsbH (Bonardi et al. 2005; Vainonen et al. 2005). STN8 is also involved in phosphorylation of other thylakoid proteins, such as the calcium-sensing receptor protein CaS and PGRL1-A, a protein probably involved in the modulation of cyclic electron transfer (Vainonen et al. 2008; Reiland et al. 2011).

Reversible phosphorylation of D1, D2 and CP43 proteins is suggested to play a role in the repair cycle of PSII (Aro et al. 1992; Baena-González et al. 1999). For more details, see Figure 3. The precise mechanism has been recently elucidated and involves control of the macroscopic structure of thylakoids (Fristedt et al. 2009b). This affirmation is based on the fact that Arabidopsis STN8-deficient plants displaying reduced PSII phosphorylation also show enhanced grana size and impairment of the PSII repair cycle (Tikkanen et al. 2008; Fristedt et al. 2009b).
1.4.3.2 STN7-mediated phosphorylation

STN7 kinase is involved in phosphorylation of proteins connected to light harvesting. It has been shown that STN7 phosphorylates subunits of LHCII, minor light-harvesting proteins CP29, CP26 and the small LHCII interacting protein TSP9 (Bellafiore et al. 2005; Tikkanen et al. 2006; Fristedt et al. 2009a). In addition, the nucleoid-associated protein pTAC16 phosphorylation is also dependent on STN7 (Ingelsson and Vener 2012).

Changes in the light environment may lead to redox changes of the PQ pool, as a part of the electron transport chain to optimize the efficiency of the photosynthetic machinery. When the PQ pool is reduced, plastoquinol (PQH2) docks to the Q0 site of Cyt b6f (Vener et al. 1997), activates STN7 (Vener et al. 1995; Zito et al. 1999); when the PQ pool is oxidized (QH2—PQ), the reverse happens. Interestingly, low light can activate STN7 kinase and LHCII is phosphorylated. However, high light inactivates the STN7 kinase, although the PQ pool is greatly reduced. This is probably due to the ferredoxin-thioredoxin system on the stromal side of the thylakoid membrane (Rintamaki et al. 1997; Rintamaki et al. 2000). STN7 was suggested to be inactivated by thioredoxin-mediated reactions in the lumen as well, because STN7/Stt7 contains two cysteins exposed to the lumen (Puthiyaveetil 2011). This proposal was supported by evidence that Stt7 kinase was inactivated by breaking a disulfide bridge between the two luminal cysteins (Lemeille et al. 2009). Mutagenesis of phosphosites of STN7 itself indicates that phosphorylation of STN7 may affect its turnover. However, phosphorylation of STN7 was not necessary for its activation in state transitions or for LHCII phosphorylation (Reiland et al. 2009; Willig et al. 2011). It has also been shown that STN8 is not required for the phosphorylation of STN7 (Ingelsson and Vener 2012).

1.4.3.3 Others phosphorylation-related enzymes

Except for STN7 and STN8 regulated phosphorylation, chloroplast casein kinase 2 (CK2)-dependent phosphorylation probably plays a major role in the chloroplast, as indicated by kinase target motif analysis (Reiland et al. 2009). The chloroplast sensor kinase (CSK) interacts with CK2 and controls the transcription of chloroplast genes based on the plastoquinone redox state (Puthiyaveetil et al. 2008; Puthiyaveetil et al. 2010). In contrast to STN8 and STN7 phosphorylation activity on PSII core and LHCII proteins, PBCP (Samol et al. 2012) and PPH1/TAP38 (Pribil et al. 2010; Shapiguzov et al. 2010) dephosphorylate these proteins, respectively.
1.4.4 Photoacclimation and state transition

Unlike animals, plants cannot move, for example, to find their desired location for photosynthesis. They have to adapt themselves to different environmental conditions, such as temperature, moisture, seasonal changes and various light intensities.

Short-term acclimation happens in minutes, whereas long-term acclimation happens in hours, days, or even longer. Short-term acclimation of plants involves a dynamic reorganization of photosynthetic complexes. Long-term acclimation involves changes in chloroplast and nuclear gene expression, which in turn alters the composition of the photosynthetic machinery for optimal performance.

State transition was traditionally proposed to be a type of short-term acclimation in plants. In state 1, PSI is preferably excited and all LHCII is associated with PSII. When PSII is preferentially excited by light, a mobile pool of LHCII moves from PSII and associates with PSI, which is defined as state 2 (Allen 1992). This process is triggered by phosphorylation of LHCII by the Stn7 kinase (Bellafiore et al. 2005). In reverse, LHCII is dephosphorylated by TAP38 (Pribil et al. 2010) or PPH1 (Shapiguzov et al. 2010). Recent reports indicated that LHCII is associated with both photosystems in natural light conditions, which is in contrast to the previous knowledge that LHCII is associated with PSI only as a short-term response (Wientjes et al. 2013a; Wientjes et al. 2013b). The weakly bonded ‘extra’ LHCII can probably be dissociated by phosphorylation (Wientjes et al. 2013a), which is consistent with the observation that in plants only about 15-20% of LHCII are found to relocate between PSII and PSI, unlike green algae, where 80% of LHCII can undergo state transition (Delosme et al. 1996).
2. SCIENTIFIC AIMS

The general aim of this thesis work is to gain more knowledge about regulatory mechanisms of photosynthesis during light stress in plants, with a focus on PSII protein phosphorylation and regulatory protein identification and characterization.

The specific aims of each project were as follows:

**Paper I:** To compare PSII performance in three widely used Arabidopsis laboratory accessions: Col-0, Ws-4 and Ler-0

**Paper II:** To investigate if natural variation in phosphorylation of PSII proteins is caused by genetic variation of STN kinases

**Paper III:** To investigate the physiological role of TAAC in Arabidopsis

**Paper IV:** To study of the membrane proteome of stroma thylakoids in order to find novel proteins with a putative role in photosynthetic regulation
3. METHODOLOGIES

3.1 Arabidopsis thaliana as a model organism

About 30 years ago, Arabidopsis thaliana, a member of the mustard family became a model organism for plant biology. The reason are its small size, short life cycle, efficient reproduction through self-progeny, and small genome size (Meinke et al. 1998). Since the complete sequencing of its genome (Arabidopsis Genome 2000), Arabidopsis research has developed significantly in the direction of functional annotation of the more than 25,000 genes (Bevan and Walsh 2005; Koornneef and Meinke 2010).

3.1.1 Natural variation

Over 7,000 Arabidopsis natural accessions are distributed and collected from all over the world (Weigel 2012). Such wide range of natural accessions differ in genetic variation including allele polymorphisms, gene duplication (Bikard et al. 2009), and also gene translocation (Vlad et al. 2010). With assistance of linkage and association mapping techniques, quantitative trait loci (QTL) will allow to identify the genomic sequence location, down to genes in Arabidopsis accessions, where they control a complex phenotypic trait (Weigel and Nordborg 2005; Flood et al. 2011; Trontin et al. 2011).

Unlike mutant-based studies, which allowed advances in certain gene functions, natural variation will provide more information about plasticity and adaptation of plants to the environment, since it is a trait of multiple gene locus contributions. The goal of crop breeders is to find breeding targets among natural variants with traits in certain environments for breeding targets (Trontin et al. 2011). Furthermore, Arabidopsis natural variation will offer a great opportunity to study plant evolutionary ecology at the molecular level (Koornneef and Meinke 2010).

3.1.2 Functional genomics

Availability of large collections of mutants greatly contributed to the emergence of Arabidopsis as model organism. About 27,000 individual gene interruption lines can be purchased at the Arabidopsis Biological Resource Centre (ABRC), which covers nearly all the protein-coding genes (http://abrc.osu.edu/about-us; http://signal.salk.edu/cgi-bin/homozygotes.cgi). This great achievement is based on small-gene-intron character in Arabidopsis. Gene function can be dramatically disrupted by insertion of a piece of T-DNA during transformation with Agrobacterium tumefaciens (Krysan et al. 1999). This means in theory that it is possible to find out the function of every gene/protein of...
interest by mutagenesis with reference inbred line-wild type. There are three commonly used laboratory wild type Arabidopsis accessions: Columbia-0 (Col-0), Landsberg erecta-0 (Ler-0) and Wassilewskija-4 (Ws-4). Due to mutant collections and AGI sequencing, Col-0 is generally viewed as the reference wild type (Koornneef and Meinke 2010).

3.2 Chloroplast fractionation

Procedures for isolation of chloroplasts, thylakoids, and grana and stroma thylakoids were described in the individual attached papers. In general, chloroplasts were isolated from homogenized Arabidopsis leaves by centrifugation and then purified by several washing steps. After that, chloroplasts were lysed by osmotic shock, and the thylakoids were separated by centrifugation (Paper I & III) or by sucrose gradient centrifugation (Paper IV). The grana and stroma thylakoid subfractions were obtained by digitonin solubilization and ultracentrifugation.

3.3 Protein identification and quantification by immunoblotting

First, proteins in a complex mixture were separated in acrylamide gels by electrophoresis according to their mobility. The smaller the size, the faster the protein moves. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which is then incubated with antibodies against the protein of interest. By detecting the linear signal of chemiluminescence from horseradish peroxidase (HRP) reaction with its substrate, which is directly linked to antibodies, it was possible to identify the protein and also determine its amount in a complex mixture. Phosphoprotein amounts were detected with phospho-threonine antibodies from both New England Biolabs (Cell signaling) and ZYMED.

3.4 Chlorophyll fluorescence

Chlorophyll a fluorescence was used to measure photosynthetic performance in our study (Paper I & III). Light energy absorbed by PSII chlorophyll can take three directions: first the energy can be used to drive photosynthesis, where electrons are generated from water splitting and transferred to the primary quinone acceptor Q\textsubscript{A}; another way is to be released as heat; or to be emitted as fluorescence. These three ways compete with each other for the energy excitation. By gaining information about fluorescence emission, we can evaluate photochemistry as well as the heat dissipation (Baker 2008). Parameters named maximum quantum efficiency of PSII photochemistry $Fv/Fm$, the quantum yield of PSII photochemistry $\Phi_{PSII}$, the excitation pressure $1-qP$, ...
non-photochemical quenching NPQ, fast kinetics (O-I-I-P curves) were recorded/calculated by either PAM-210 or Dual PAM-100 in Paper I and III.

3.5 Mass spectrometry

In the last decades mass spectrometry (MS) has become invaluable method in a large-scale protein identification and characterization. In a classical MS procedure, a complex peptide mixture or other macromolecules are first separated by liquid chromatography (LC) based on reversible hydrophobic interaction with stationary phase of the chromatographic medium before subjection to ionization.

There are two major ionization methods: electron spray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). MALDI produces ions by laser excitation, the peptides are singlet charged and usually conjugated with time of flight (TOF) analyzer. Different from MALDI, ESI can produce multiply charged ions, which is usually coupled with tandem mass spectrometry (ESI-MS/MS) that can detect the amino acid sequence of the peptide fragment. The Noble Prize 2002 in Chemistry was awarded to John B. Fenn and Koichi Tanaka for development of these ionization methods (Tanaka et al. 1988; Fenn et al. 1989).

MS/MS fragments of charged ions are obtained by collision-induced dissociation (CID) and electron transfer dissociation (ETD). CID is most commonly used peptide fragmentation method for amino acid sequence determination (Mann et al. 2001). ETD is more able to preserve posttranslational modification of peptides as compared to the more crude CID technique (Syka et al. 2004). We have been using ESI followed by CID and ETD (HCTultra PTM Discovery System, Bruker Daltonics) to identify regulatory proteins in thylakoid membrane in Paper IV.

3.5.1 In proteomics

Proteomics, the large scale study of proteins, has become an important tool to achieve this aim by several aspects: identification and characterization of known and hypothetical proteins with different post-translation modifications in a certain subcellular compartment; comparison of protein composition in several environmental conditions, for example light-stressed and dark-adapted plants; studies of protein-protein interaction with method such as pull-down experiments and mass spectrometry (Pandey et al. 2000; Ephritikhine et al. 2004).

Chloroplast, the photosynthetic organelle, contains photosynthetic core and regulatory proteins. Proteomic study of chloroplast subfractions will provides comprehensive information about regulatory proteins. In the last ten years, several independent laboratories have conducted proteomic analysis on chloroplast envelope (Ferro et al. 2003; Froehlich et al. 2003; Rolland et al. 2003), thylakoid membrane and lumen (Peltier et al. 2002; Schubert et al. 2002; Friso et al. 2004). Most of the identified chloroplast proteins can be found in two databases of the chloroplast proteome from

About 3600 distinct proteins are predicted in Arabidopsis chloroplasts (Abdallah et al. 2000; Kleffmann et al. 2004). However, only about 1,300 proteins were verified in AT_Chloro and 2300 are curated at PPDB. The reasons for the difference between the number of predicted and identified chloroplast proteins could be the following: low abundance; expressed only under certain conditions (for example abiotic stress); the tryptic peptides are not ionized. To be able to improve the identification rate, more sensitive and accurate mass spectrometry, more specific fractionation of chloroplasts and alternative enzyme digestion have been suggested to be employed in the investigation (van Wijk and Baginsky 2011).
4. MAJOR FINDINGS

4.1 Impact of thylakoid lipid-chlorophyll-carotenoid stoichiometry on PSII function

Few studies have been previously carried out to analyze natural variation of photosynthetic parameters in Arabidopsis, namely thermal dissipation of excess light energy (Jung and Niyogi 2009) and Rubisco small subunit diversity (Schwarte and Tiedemann 2011). In other species, for example in maize, variation in cold tolerance of photosynthesis was studied (Fracheboud et al. 2002).

Col-0, Ws-4 and Ler-0 are three widely used Arabidopsis background accessions to characterize the relevant mutant lines. In Paper I, I found that Ws-4 is more susceptible to high light as compared to Col-0 and Ler-0. It displayed higher energy fluxes through PSII reaction center, and also faster closure upon both saturation pulse and continuous illumination, as indicated by fluorescence measurements such as J fluxes, $O-J-I-P$ induction and rapid light curves of $\Phi$PSII, $1-qP$. These observations could be explained by approx. 30% higher thylakoid lipid-to-Chl ratio and 40% lower Chl/carotenoid ratio in Ws-4 as compared to Col-0 and Ler-0. A higher thylakoid lipid-to-Chl ratio means that the Chl-protein complexes are more ‘diluted’ with lipids in the leaves of this accession. This could result in faster closure of RCII centers in Ws-4.

The content of two major types of lipids (MGDG and DGDG) is higher in Ws-4. Unlike DGDG, MGDG is a non-bilayer lipid that can create a lateral pressure within the hydrophobic membrane (van den Brink-van der Laan et al. 2004). How does Ws-4 solve the problem with the lateral pressure? The lower Chl-to-carotenoid ratio in Ws-4, especially due to higher violaxanthin content, means that a fraction of additional free violaxanthin may function as compensation for additional MGDG causing lateral pressure on the membrane. Another fraction of the additional violaxanthin may bind to LHCII-PSII supercomplexes to participate in RCII excitation. It has been shown that the polarity of xanthophyll bound to LHCII increases the PSII quantum efficiency because the lifetime of the Chl excited state is prolonged (Ruban and Johnson 2010).

4.2 Impact of STN8-chlorophyll-lipid stoichiometry on PSII dynamics

In Paper I, I also found that HL treatment caused in Ws-4 the largest extent of PSII inactivation and fastest D1 degradation. Based on further investigation using blue native gel electrophoresis and immunoblotting we demonstrated that in this accession PSII undergoes faster disassembly of PSII-LHCII supercomplexes.
It has been suggested that STN8 is essential in controlling the lateral movement of PSII-LHCII supercomplexes along the thylakoid membrane to the sites of repair since the \textit{stn8} mutant displayed residual D1/D2 protein phosphorylation, more damaged supercomplexes, and delay in D1 protein degradation (Pesaresi et al. 2011; Tikkanen and Aro 2012). Based on this thesis work, Ws-4 doesn’t show an intermediate D1 turnover because of its intermediate phosphorylation level. Instead D1 protein is degraded faster than in the other two accessions. I observed lower STN8-Chl ratio in Ws-4 that could explain the reduced phosphorylation. This indicates that variation in STN8 levels may not be the only factor involved in the lateral movement of supercomplexes, in contrast with previous single-gene-mutation studies. In Ws-4 the dilution of protein complexes by additional lipids plays a major role in disassembly and migration of the damaged complexes to the sites of repair, thus compensating for consequence of intermediate phosphorylation levels of the PSII core proteins. Although PSII complexes may move faster in the thylakoid membrane for a faster D1 degradation, additional PSII damage occurs in Ws-4, which exceeds the D1 protein synthesis capacity, thus leading to slightly enhanced photoinhibition.

4.3 Variation of PSII protein phosphorylation in natural accessions

Enzymes named kinases catalyze phosphorylation of proteins. PSII core proteins D1, D2 PsbH and to some extent CP43 proteins can be phosphorylated by STN8 kinase under HL conditions (Vainonen et al. 2005); whereas STN7 is involved in phosphorylation of LHCII proteins under growth light (GL) conditions (Bellaﬁore et al. 2005). Since PSII protein phosphorylation is an important regulatory mechanism used by plants to adapt to various light intensities and qualities, our next question was to investigate if natural accessions differ in PSII protein phosphorylation and the reasons behind their variation. In Paper II, 16 Arabidopsis accessions from diverse geographic origins were exposed to two light regimes, and the levels of phospho-D1 and phospho-LHCII proteins were quantified by immunoblotting with anti-phosphothreonine antibodies. The levels of STN7 and STN8 kinases were also tested. D1/Lhcb2/CP43 protein levels were used to correct levels of corresponding phosphorylated protein and of STN kinases (Figure 4).

4.3.1 Role of kinase activity under growth light

From our natural accessions selection, Col-0 shows the highest D1 phosphorylation in GL although the others display similar abundance of the D1 substrate and STN8 kinase. One explanation could be a poor activation of the STN8 kinase in GL, which can be related to the redox state of the PQ pool. The same goes for the STN7 kinase, i.e., it is rather STN7 activity than protein level that regulates the phosphorylation of LHCII under GL conditions.
Figure 4. Quantitative immunoblotting analysis of phospho-D1, STN8, phospho-LHClI and STN7 levels (A), and control blots with D1, Lhcb2 and CP43 level (B) in thylakoids from natural accessions, the stn8 mutant and the hybrid line Tsu-0 x Ws-4 (TxW).

Among the accessions we examined, Ely and ELB are negative controls for kinase activity. Both of them are atrazine resistant due to deficient binding of quinones in the Q_B pocket (El-Lithy et al. 2005). Low phosphorylation of PSII core and LHCII proteins were detected in both accessions, which could be due to poor reduction of PQ pool, hence reduced activation of the STN kinase.

4.3.2 Role of kinase abundance under HL

PSII protein phosphorylation levels can be under the control of kinase amount. In paper II, figure 2 b we found that the levels of D1 phosphorylation correlated with the STN8 kinase levels in HL, supporting the initial observations in Paper I. This indicates that kinase abundance can be a limiting factor or play a regulatory role for phosphorylation under these conditions.

4.3.3 Role of protein phosphorylation in adaptation to diverse environments

We propose that variable phosphorylation of PSII proteins in Arabidopsis accessions is a result of an adaptive mechanism for survival and reproduction across the range of environmental conditions where Arabidopsis naturally occurs. In Paper II, we found a significant correlation between longitude and both D1 and LHCII phosphorylation in GL conditions (figure 4 a, b). This suggests that there may be some form of selective pressure that correlates with longitude.

When comparing phosphorylation levels of PSII proteins with climate data from WorldClim database (Hijmans et al. 2005) (http://www.worldclim.org/), we found positive correlation between phospho-LHCII in GL with temperature seasonality and with temperature annual range to be significant. It may be the result of similar photoprotective mechanisms as observed in evergreen trees to maintain functional
leaves in very cold conditions (Verhoeven et al. 2009). To investigate correlation between phosphorylation and the natural habitat, many more accessions should be included from a large range of environments.

### 4.4 Physiological roles of the thylakoid ATP/ADP carrier in Arabidopsis

Thylakoid ATP/ADP carrier (TAAC) belongs to the mitochondria carrier family. It is absent in cyanobacteria and only appeared early in the evolution of photosynthetic eukaryotes (Palmieri et al. 2011; Spetea et al. 2011). Previous study from our laboratory have shown that TAAC protein imports ATP in exchange for ADP across the cytoplasmic membrane of Escherichia coli, and that the taac mutant thylakoids have reduced ability to take up ATP and convert it to GTP as compared to the wild-type (Thuswaldner et al. 2007). **Paper III** is focused on the physiological consequence of TAAC protein deficiency in Arabidopsis.

#### 4.4.1 Role in PSII repair

The deficiency of TAAC in the mutant has increased the susceptibility of PSII to photoinhibition under both HL conditions studied: 3h illumination and 4 weeks in the growth chamber (**Paper III**). Detailed phenotypic investigation showed that PSII repair cycle is malfunctions in the mutant as compared with background wild type, which is the primary reason of higher susceptibility of PSII to HL. The site of malfunction was localized at the step where CP43 dissociated from damaged PSII, since a relatively low proportion of CP43-less monomer was detected by blue native gel electrophoresis together with immunoblotting in the mutant compared with the wild type.

Interestingly, previous studies of the psbo2 mutant showed a similar phenotype as for the taac mutant in PSII repair aspects during HL stress. Both types of plants were smaller and less tolerant to HL conditions, displayed retarded D1 protein degradation, and blockage in PSII repair cycle step from core monomer to CP43-less monomer (Lundin et al. 2007a; Lundin et al. 2008). PsbO2 was demonstrated as a GTPase (Lundin et al. 2008), which activity is required to induce its dissociation from PSII thus also facilitating CP43 dissociation from PSII core monomer. Our hypothesis in **Paper III** was that TAAC and PsbO2 crosstalk in the CP43 dissociation step as reviewed (Spetea and Lundin 2012). In addition, ATP transported via TAAC into the lumen may induce phosphorylation of PSII lumen-exposed proteins with role in efficient turnover of the D1 protein as illustrated in **Figure 5**. Briefly a) ATP is transported by TAAC into the thylakoid lumen in exchange for ADP (Thuswaldner et al. 2007); b) ATP is then converted to GTP, which binds to the PsbO subunit of PSII complex, resulting in monomerization; c) PSII monomer moves to stroma lamellae, GTP is also used to dissociate PsbO2 from PSII monomer to CP43 less monomer (Lundin et al. 2007b); d) An unknown kinase phosphorylates CP43 lumenal-exposed loop and lumenal proteins,
leading to their dissociation from PSII/membrane (Spetea and Lundin 2012); e) The phosphorylated proteins are then dephosphorylated by a phosphatase, such as TLP18.3 (Wu et al. 2011), and the resulting phosphate is exported out to the stroma by the thylakoid phosphate transporter (PHT4;1) (Pavon et al. 2008).

**Figure 5.** Working model for TAAC involvement in PSII repair cycle. This involvement happens at several steps: a) ATP is transported into thylakoid lumen in exchange of ADP by TAAC; b) ATP is then converted to GTP, which binds to PsbO subunit of PSII complex, resulting in its dissociation; c) PSII monomer moves to stroma lamellae, GTP is also used to dissociate PsbO2 from PSII monomer to CP43 less monomer; d) An unknown kinase phosphorylates CP43 lumena- exposed loop and luminal proteins (PsbP, PsbQ, PsbR, Deg1 and Cyp38), leading to their dissociation from PSII/membrane; e) The phosphorylated proteins are then dephosphorylated by a phosphatase, such as TLP18.3, the resulting phosphate (P) is exported out to the stroma by the thylakoid phosphate transporter (PHT4;1). Figure modified from Spetea and Lundin (2012) FEBS Lett. 586: 2946-2954 with kind permission from the authors.

### 4.4.2 Role in PSII photoprotection

The taac mutant displayed a higher initial phase in induction of NPQ as compared to wild type, but similar steady state NPQ values. This pattern was preserved in dithiothreitol (DTT)-pretreated leaves, but not in nigericin-pretreated ones. DTT is an inhibitor of violaxanthin de-epoxidation, whereas nigericin is a common inhibitor of
trans-thylakoid pH gradient. The transiently higher NPQ in the mutant originates from higher pH gradient as compared to wild type.

A potential explanation for this result is that TAAC imports \( \text{ATP}^+ \) in exchange for \( \text{ADP}^+ \) across the thylakoid membrane. To compensate for the extra-charge, TAAC transport capacity possibly consumes a fraction of the \( \text{H}^+ \) gradient. In the mutant, this fraction can be used for energy dissipation, resulting in higher initial phase of NPQ as compared to the wild type.

### 4.4.3 Other roles in plants

Besides a role in trans-thylakoid adenine nucleotide exchange (Paper III, Thuswaldner et al. 2007), a recent published paper suggested that TAAC could be located in the envelope supplying plastidic phosphoadenosine 5’-phosphosulfate to the cytosol (Gigolashvili et al. 2012). The rice homologue of TAAC was dually localized to mitochondria and envelope (Xu et al. 2013). In the same report, the protein functioned as a 3’-phosphoadenosine 5’-phosphosulfate (PAPS) carrier, having role in chloroplast retrograde signaling and chloroplast development in rice.

### 4.5 The grana ultrastructure limits D1 turnover rate

Previously, it was reported an increased size in Arabidopsis grana thylakoids, and retarded D1 degradation in stn8 mutants as compared to the wild type (Tikkanen et al. 2008; Fristedt et al. 2009b). Furthermore, thylakoid membrane fluidity was suggested to facilitate access PSII complexes to stroma-exposed active protease FtsH, leading to efficient D1 turnover (Yoshioka and Yamamoto 2011). These two findings indicate there is direct connection between thylakoid ultrastructure and D1 turnover rate.

These observations were confirmed in Paper III. Under 3h HL illumination, TAAC deficient mutant showed increased width and height of grana thylakoids in comparison with the wild type, leading to slower PSII repair. One possible explanation is that deficiency of nucleotides in the lumen has a similar impact on stacking as the lack of PSII protein phosphorylation on the stromal side of the thylakoid membrane.

In Paper I, Ws-4 displayed smaller grana and more unstacked thylakoids most likely due to higher content of lipids per protein complexes that may have increased membrane fluidity. This has resulted in faster repair despite the reduced levels of PSII core protein phosphorylation.

### 4.6 Proteomic study explores thylakoid proteins with regulatory roles

In Paper IV, 58 proteins have been identified in the stroma thylakoid membrane of Arabidopsis thaliana. Four different methods for extraction of peptides from
hydrophobic membrane proteins have been used: in solution digestion with chymotrypsin alone and in combination with methanol extraction, in-gel digestion with trypsin and in-gel digestion with chymotrypsin. Using these methods, I was able to extract peptides from 34, 49, 5 and 6 proteins, respectively. The combination of methanol and chymotrypsin was found as the most effective way to identify membrane proteins (45 out of 58). Using a combination of prediction programs ChloroP, TargetP, and Wolf PSORT, about 50% the identified nuclear-encoded proteins were indicated as chloroplast proteins. In addition, 13 chloroplast-encoded proteins were found.

With respect to the functional classification, 25 of the identified proteins are subunits of photosynthetic complexes, nine are transporters, three have a putative role in protein fate (proteolysis, import), four in translation, two are stress-related proteins, three are involved in primary and secondary metabolism, two are related to signaling, and ten are probable contaminants. Among the 191 unique peptide sequences identified, there were 19 peptide sequences from transmembrane regions of photosynthetic proteins and transporters. This means that I have found a simple and fast strategy to extract peptides from hydrophobic low abundant membrane proteins. The obtained proteomic data require validation by individual localization studies and phenotypic characterization of mutant lines.

Figure 6. Overview of the stroma thylakoid proteome. Major photosynthetic complexes PSII, Cytb6f, PSI, ATP synthase were found in the thylakoid membrane. Besides this, proteins that function in transport, protein fate, translation, signaling, stress, and metabolism, were identified. In addition to putative channels and transporters identified by proteomics, the thylakoid ATP/ADP carrier (TAAC) and the thylakoid phosphate transporter PHT4;1 found by immunoblotting are shown.
5. CONCLUSIONS AND PERSPECTIVES

No previous studies investigated the natural genetic variation in plant photosynthesis with focus on PSII performance and dynamics. After this thesis work, we now know that out of three widely used reference accessions Ws-4 undergoes faster PSII repair but is also more susceptible to HL, mainly due to dilution of Chl-protein complexes by lipids and carotenoids in the thylakoid membrane and reduced STN8 kinase levels (Paper I). When investigating larger number of natural accessions, I found that in high light conditions phospho-D1 level is correlated with STN8 kinase amount, whereas in growth light it is correlated with longitude, and in the case of LHCII phosphorylation also with temperature variability, indicating adaptation to diverse environments for Arabidopsis (Paper II). Previously TAAC has been characterized biochemically to import ATP in exchange for ADP in Escherichia coli and Arabidopsis. But the physiological role of TAAC in plants was not clear. After characterization of taac mutants, we now postulate that TAAC plays a critical role in disassembly steps during PSII repair and in addition may balance the trans-thylakoid electrochemical H⁺ gradient storage (Paper III). TAAC is not the only regulatory protein for adaptation of photosynthetic process to various environments. In this thesis work, stroma thylakoid membrane proteomics in Arabidopsis has revealed a large number of low-abundant hydrophobic proteins, like transporters, which help us to better understand photosynthetic metabolism at molecular level and provides biochemical overview of chloroplast membranes (Paper IV).

Photosynthesis is vital for all the living organisms on Earth, however the molecular regulatory mechanisms behind are far from being completely understood. The findings presented in this thesis add stones on the castle how plants optimize photosynthesis to tackle environmental stress. By improving the knowledge about the regulatory factors involved, we will not only be able to improve our food production, but also mimic natural photosynthesis to generate renewable energy to meet our increasing demands in the future.
6. POPULÄRVETENSKAPLIG SAMMANFATTNING

Fotosyntes är den process där växter, alger och cyanobakterier använder solenergi för att omvandla vatten och koldioxid till syre och energi i form av kolhydrater. Syre är livsnödvändigt och kolhydrater ger energi direkt eller indirekt via näringskedjan till levande organismer på jorden, inklusive människan.

Solljus är viktigt för att driva fotosyntesen, men det är också känt att alltför mycket ljus kan skada de proteiner som krävs i fotosyntesen, vilket påverkar växternas tillväxt. Växter kan inte röra sig som djur, exempelvis för att hitta sin önskade plats för fotosyntesen. De måste anpassa sig till olika miljöförhållanden, såsom temperatur, fukt, ljusintensitet och årstidsväxlingar. I denna avhandling har jag använt modellväxten Arabidopsis thaliana för att studera de molekylära mekanismer som reglerar fotosyntesen under hög ljusstress.


En annan mekanism som växter använder för att övervinna hög ljusstress är PSII:s reparationscykel. I denna process är fosforylering ett tidigt och avgörande steg eftersom det hjälper skadade PSII-komplex att förflytta sig till platser i tylakoidmembranet där de kan repareras. I Artikel I har jag funnit att fosforylvingsgraden hos Arabidopsis ekotyp Ws-4 är hälften så hög jämfört med andra ekotyper, såsom Col-0, vilket beror på lägre förekomst av STN8-kinas. Som kompensation för detta har Arabidopsis Ws-4 ytterligare lipider och karotenoider som kan öka rörligheten hos de skadade komplexen och på så sätt möjliggöra repairation.

I tylakoidmembranet finns ett vätskefyllt utrymme som kallas lumen. TAAC (thylakoid ATP/ADP carrier) transporterar ATP, som används för energiöverföring, in i lumen i utbyte mot ADP. I Artikel III har jag upptäckt att planter som saknar TAAC har långsammare PSII-reparation än planter med TAAC, trots att de uppvisar samma grad av proteinfosforylation. Vi föreslår att ATP, som transporterats in i lumen av TAAC, används för nukleotidberoende reaktioner som påverkar PSII-reparationen. En annan upptäckten är att TAAC via sin transportaktivitet kan förbruka en del av protongradienten över tylakoidmembranet, vilket är avgörande för initieringen av ljusskyddande mekanismer.
I Artikel IV har jag identifierat 58 proteiner, som inkuderar såväl tidigare kända som nya tylakoidproteiner med potentiella roller i transport, translation, stress och signalering. Denna avhandling fördjupar vår förståelse av fotosyntesen på molekylär nivå och förbättrar den biokemiska översikten av tylakoidmembranet i kloroplasten.
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